Vascular Tumor Necrosis Factor-alpha Gene Expression in Human Aortic Valve Calcification

Expression of TNF-α in Calcified Aortic Valves

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Abstract

The intimate mechanisms involved in aortic valve calcification (AVC) are not completely known. It is more evident to suggest that cardiovascular calcification is an inflammatory condition. Tumor necrosis factor alpha (TNF-α), a pro-inflammatory cytokine, is increased in valvular lesions besides areas of leukocyte infiltration. The aim of this study was to test the hypothesis that valvular calcification is part of a generalized inflammatory process involving the vascular wall. The differential gene expression of TNF-α in thoracic aortic samples from 49 patients with AVC has been assessed in comparison with samples from 94 patients with non-calcified valves. As compared with subjects without calcification, patients with AVC were older (P<0.01) and had an increased prevalence of coronary atherosclerotic disease (P=0.002), left ventricular hypertrophy (P<0.001), smoking habit (P=0.027) and hypertension (P=0.05). The incidence of AVC was significantly higher in smokers, hypertensives, and patients with coronary atherosclerosis. All thoracic aorta samples showed expression of TNF-α. The mRNA expression level of TNF-α was significantly higher in patients with AVC (P<0.01). Likewise, a significantly higher expression level of TNF-α was found in subjects with atherosclerotic coronary disease (P<0.01). Insignificant influence was detected for the effect of other variables, including smoking, diabetes, hypertension or gender. In conclusion, the present study supported the notion that valvular calcification is part of an inflammatory-based process affecting the vascular system.

Keywords

Aortic Valve; Calcification; Inflammation; Gene Expression

Introduction

Calcific aortic stenosis is the most common cause of aortic valve disease in western population (Anger et al., 2009). The intimate mechanisms involved in aortic valve calcification (AVC) remain unclear, resulting in the lack of appropriate therapies to prevent the development and progression of this process. Alteration in the leaflet cell biology results in calcification on the aortic surface of the valve cusp (Warren et al., 1997), with impaired movement of the leaflets that can lead to heart failure and death (Otto et al., 1999).

Several studies have provided data supporting the concept of cardiovascular calcification as an inflammatory disease (Lee et al., 2011; Mohler et al., 1999; Yu et al., 2011), instead of a passive accumulation of calcium on vascular beds. Therefore, both cardiovascular risk factors and atherosclerosis are thought to be involved in a process of osteogenic differentiation (Anger et al., 2009), constituting a developing force for endothelial dysfunction and calcification (Goldbarg et al., 2007; Tanaka et al., 2005).

Experimental studies have shown an increased expression of the pleiotropic cytokine tumor necrosis alpha (TNF-α) in valvular lesions besides areas of leukocyte infiltration (Al-Aly et al., 2007). Furthermore, this inflammatory cytokine plays a regulatory role in the remodelation of the extracellular matrix (Kaden et al., 2007) and in the induction of osteogenic differentiation and mineralization of vascular cells (Tintut et al., 2000).

The purpose of this study is to test the hypothesis that valvular calcification is part of a generalized inflammatory process involving the vascular wall, in which TNF-α plays a key role. The differential gene expression of TNF-α both in thoracic aorta samples from patients presenting AVC and in subjects with non-calcified valves have been assessed.

Materials and Methods

Patients

Thoracic aorta specimens were obtained from 143 consecutive patients who were underwenting elective coronary artery bypass or valve replacement surgery. Informed consent was obtained from all patients according with Helsinki rules, and the study protocol was approved by the local Ethics Committee.

The clinical and demographic data of the patients were achieved and clinical records were reviewed for cardiovascular risk factors, including smoking, hypertension, dyslipidemia, and diabetes, as well as for a history of peripheral vascular disease. Coronary atherosclerosis was determined by standard coronariography, and left ventricular hypertrophy and AVC were assessed by 2-dimensional transthoracic echocardiography. Image studies were analysed blinded to all clinical details of the study patients.

Gene expression

Tissue samples were immediately placed in RNAlater® (Ambion (Europe) Limited, UK) solution and stored at 4°C for subsequent RNA extraction. Total RNA was isolated from tissues kept on ice after complete homogenization in TRI Reagent® (Sigma-Aldrich, St. Louis, MO, USA) employing TissueRuptor (Qiagen, Hilden, Germany) and further purified using RNeasy Mini kit (Qiagen). Quality of extracted RNA was tested using an ExperionTM Automated Electrophoresis System (Bio-Rad Laboratories, Hercules, CA, USA) to ensure that 28S and 18S rRNA bands were clearly evident. RNA was quantified using a Thermo Scientific NanoDrop 2000 spectrophotometer (Thermo Scientific Nanodrop, USA). The cDNA was obtained using a High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA). Transcripts were measured by TaqMan real-time quantitative PCR (qRT-PCR) with TaqMan Fast Universal PCR Master Mix (Applied Biosystems). TaqMan Gene Expression Assays for each transcript (Hs00174128m1 [TNFα] and Hs99999905m1 [Glyceraldehyde 3-phosphate dehydrogenase, GAPDH]) were analysed in a 7500 Fast Real-Time PCR System (Applied Biosystems). The level of target mRNA was estimated by relative quantification using the comparative method (2-$\Delta\Delta$Ct) by normalization to GAPDH expression. Quantification of each cDNA sample was tested in triplicate, and a corresponding non-reverse transcriptase reaction was included as a control for DNA contamination.

Statistical Analysis

Continuous variables are expressed as means ± standard deviation (SD) or median and range. Dichotomous variables are expressed as numbers and percentages. Between-group comparisons were tested using the Mann-Whitney test and chisquare test as

appropriate. The fold-change in the expression of the TNF-α gene was calculated with Data AssistTM v2.0 Software (Applied Biosystems) and results were exported to SPSS 19.0 for further analysis. Statistical significance was established at P<0.05.

Results

There were 101 men and 42 women, with a mean age of 64 ± 11 years (range 22-83). Regarding the prevalence of cardiovascular risk factors, 62% had hypertension, 60.8% dyslipidemia, 51% left ventricular hypertrophy, 44.1% antecedent of tobacco use, and 40.5% diabetes mellitus (Table 1). Atherosclerotic coronary disease was present in 62.9% of patients, whereas AVC was observed in 38 patients (26.5%).

The mean age of patients with AVC was significantly higher (P<0.01) than that in subjects without calcification. As compared with subjects without calcification, patients with AVC had an increased prevalence of coronary atherosclerotic disease (P=0.002), left ventricular hypertrophy (P<0.001), smoking habit (P=0.027) and hypertension (P=0.05), whereas there were no differences regarding dyslipidemia or diabetes (Table 1). The incidence of AVC was significantly higher in smokers (43%), hypertensives (69.3%) and patients with coronary atherosclerosis (46.9%). There was no difference in the age of patients with or without coronary atherosclerosis (64 ± 11 vs 64 ±13).

When the potential influence of genderwas analyzed, a similar prevalence of AVC was observed in men and women (23.8% and 33.3%, respectively). On the contrary, the incidence of coronary atherosclerosis was higher in men (68.3% vs 21.5%, respectively). Male patients had a significantly higher prevalence of hypertension (P<0.001) and diabetes (P<0.05). Finally, the effect of hypertension on the prevalence of AVC was observed only in male patients.

Quantitative analysis of TNF-α expression

Gene expression of TNF-α was detected in all vascular wall samples. The mRNA expression level of this cytokine in patients with AVC (1.137 [0.997-2.034] arbitrary units [a.u.]) was significantly higher than that in patients without valve calcification (0.983 [0.424 - 1.17] a.u.) P<0.01) (Figure 1). Likewise, it was found that male patients with coronary atherosclerosis showed a statistically significant higher expression level of TNF-α than subjects without coronary disease (1.87 [0.82 - 2.12] vs. 0.85 [0.21 - 1.02] a.u.; P<0.01). However, these differences was not observed in female patients. Insignificant differences in the TNF-α expression levels were observed for the effect of other variables, including smoking, diabetes, hypertension or gender.

TABLE 1 PATIENTS DEMOGRAPHICS

Characteristics	All subjects	With Calcification	Without Calcification	*P*
Patients, n	143	38	105	
Sex, Male/Female	101/42	24/14	77/28	0.238
Age, years	64±11	70±9	62±3	<0.01
Smoking, %	44.1	39.5	26.4	0.027
Dyslipidemia, %	60.8	63.2	61.8	0.733
Diabetes, %	40.5	39.5	38.6	0.874
Coronary atherosclerosis, %	62.9	42.1	36.1	0.002
Left ventricular hipertrophy, %	51	36.8	10.5	<0.001

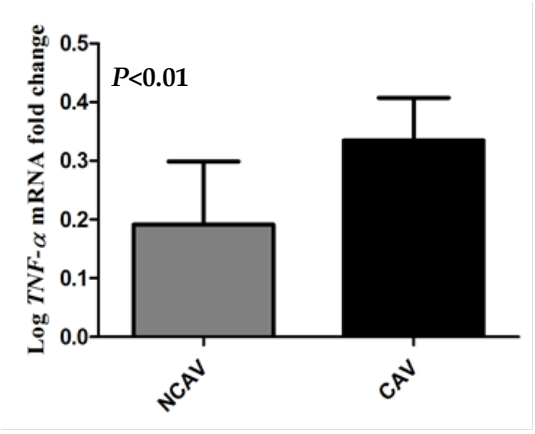

FIG. 1 QUANTITATIVE RT-PCR MEASUREMENT OF TNF-A MRNA IN THORACIC AORTA TISSUE SAMPLES FROM SURGERY PATIENTS. BARS REPRESENT MEAN VALUES. CAV: CALCIFIC AORTIC VALVES; NCAV: NOT CALCIFIED AORTIC VALVES

Conclusion

This study provided evidence that the vascular wall of patients with AVC showed an increased gene expression of the inflammatory cytokine TNF-α. The presence of inflammatory cells, lipoproteins and bone matrix proteins in the calcified regions of the cardiac valves (Mohler et al., 1999), along with the association with common cardiovascular risk factors, suggested that valvular calcification is part of a generalized process involving the vascular wall dependent on common pathogenetic inflammatory mechanisms. Therefore, it would appear appropriate to consider valvular calcification a hallmark of vascular disease.

The influence of traditional cardiovascular risk factors for vascular atherosclerosis on the development and progression of aortic valve calcification has been shown previously (Stewart et al., 1997). In our study, smokers, hypertensive and atherosclerotic patients had a statistically significant higher incidence of AVC, whereas patients with AVC had an increased prevalence of coronary atherosclerotic disease, left ventricular hypertrophy, smoking habit and hypertension.

Based on the presence of inflammatory cells and atherosclerosis in early aortic valve lesions, as well as the demonstration of inflammatory molecules in valve specimens from patients with aortic disease, AVC has been considered as an inflammatory condition (Rajamannan et al. 2007). Experimental studies have reported that the inflammatory cytokine TNF-α is abundantly present in areas of leukocyte infiltration in stenotic aortic valves (Al-Aly et al., 2007). In addition, in vitro studies have shown that valvular calcification is actively regulated by an inflammatory process involving TNF-α (Tintut et al., 2000; Kaden et al. 2005). The results of our study have demonstrated that TNF-α is significantly overexpressed in thoracic aorta of patients with AVC as compared with subjects without valvular calcification, and have provided new evidence on the similarity between valvular and vascular disease, supporting the hypothesis that valvular calcification is part of a generalized inflammatory process involving the vascular system. Further, our findings open new intriguing questions about the task of TNF-α in AVC, such as the potential effect of this cytokine as a paracrine or autocrine factor released by the vascular wall, the role of other components of the TNF-α system (TNF-α receptors or TNF-α converting enzyme) on valvular calcification, or the position of the TNF-α system as a therapeutic target for treatment of AVC.

Although novel information has been presented, it is acknowledged that there are several limitations to this investigation. First of all, the relatively small sample size allowed control only over a limited number of confounders. Secondly, the cross-sectional nature of the study does not allow causal inferences. The last but not the least, a histological study lacks on the location of TNF-α in the vessels of these patients. Nonetheless, combined with findings from previous works, our study supports the notion that valvular calcification is part of an inflammatory-based process affecting the vascular system.

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